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Edward A. Dratz, Ph.D.

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Bacteriorhodopsin is the best understood transmembrane ion pump. Bacteriorhodopsin creates ca. 100 mV transmembrane potential by pumping protons across its membrane when illuminated by visible light. Visible light isomerizes a small (ca. 300 Dalton) chromophore called retinal which drives protein conformational changes that accomplish the pumping. Pumping occurs in a series of steps, and the intermediate forms can be trapped for study at sufficiently low temperatures. New methods of solid state NMR have recently provided striking new information on the detailed structure of the retinal active site in bacteriorhodopsin. However, the light induced changes that produce transmembrane proton pumping have not been studied. Experiments underway are designed to reveal the light induced conformational changes at the active site, the light induced charge movements, and the coupling of the charge motion to the active site conformational changes in bacteriorhodopsin using new solid state NMR methods.

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Progress Report on Contract N00014-87-K0278

Principal Investigator: Edward A. Dratz, Montana State University

Title:

Mechanism of Conversion of Light Into Chemical Energy in Bacteriorhodopsin: Identification of Charge Movements and Coupling to Active Site Conformational Changes

Date: June 30, 1988

The goal of this project is to understand the mechanism of light energy capture by bacteriorhodopsin (bR) and its coupling to transmembrane proton pumping. Bacteriorhodopsin is able to generate approximately a -150 mV electrochemical gradient of protons across a 3.0 nm membrane hydrocarbon permeability barrier. The electrical component of this gradient can be as large as 50,000 V/cm. The pumping process uses energy captured from light to drive a photochemical cycle of bR intermediates (J, K, L, M and O) to accomplish the transmembrane pumping process. We are using new methods of solid state NMR to study structural changes in both the protein and its photochemical intermediates. This work will provide a detailed molecular mechanism by which bacteriorhodopsin converts light into electrochemical energy.

Light is absorbed by a chromophore, all-trans retinal, bound to the protein by a Schiff base linkage at lysine-216 in bacteriorhodopsin. After light absorption, the retinal undergoes a fast conformation change to a 13-cis configuration. The subsequent return to the all-trans configuration causes structural changes in the bacteriorhodopsin retinal-protein complex which, in turn, drive the energetically "uphill" pumping process. Solid state NMR has been shown to provide abundant new information on the retinal conformation while in the all-trans ground state configuration (1). We have begun to apply solid state NMR techniques to the active pumping intermediates of bacteriorhodopsin.

In order to take advantage of the enormous information content available in solid state NMR, isotopic labels must be incorporated at specific sites. We are primarily using C-13 labeling, but F-19 labeling will also be a powerful tool for these studies. The C-13 labels can be incorporated at specific sites in retinal. These molecules have been prepared by chemical synthesis in collaboration with Prof. Koji Nakanishi's group at Columbia University, New York. We incorporate the labeled retinal into bacteriorhodopsin by removing the native retinal, i.e., by bleaching with light and hydroxylamine, and then reconstituting with the C-13 retinal. F-19 is incorporated in a similar way using labeled retinals synthesized by Prof. Robert Liu at the University of Hawaii, Honolulu, Hawaii.

Isotopic C-13 labels can also be incorporated into the carboxyl groups of aspartic acid in bacteriorhodopsin using two very different methods. The aspartic acid sites are of interest because FTIR spectroscopy and site-specific mutagenesis experiments (2,3) indicates that these groups are essential for carrying protons across the membrane during pumping. The first method uses in vivo biosynthetic incorporation of labeled aspartic

acid by the bacteria. This labels all the aspartic acid sidechains of the protein. Our work on optimization of this method will be discussed below. The second method uses in vitro semisynthesis of parts of the bacteriorhodopsin protein to label only single aspartic acid sites. This work is in collaboration with Prof. Robert Renthal at the University of Texas, San Antonio.

To successfully obtain the desired information, several steps are needed:

1. Obtain high yield, efficient incorporation of isotopic labels into functional bacteriorhodopsin.

2. Produce large amounts of essentially homogeneously labeled bacteriorhodopsin for study.

3. Accomplish measurement of solid state CPMAS NMR spectra of C-13 labeled molecules and MAS spectra of F-19 labeled molecules at both ambient and low temperatures.

4. Deduce the chemical shift anisotropy (CSA), i.e., the chemical shifts in three directions in space around each labeled site, from the measured C-13 and F-19 data.

5. Obtain the solid state NMR spectra of isolated photointermediates using low temperature trapping or pulsed laser excitation with synchronized NMR data acquisition.

6. Carry out a systematic investigation of the NMR properties of labeled retinal and aspartic acid sites in bacteriorhodopsin pumping intermediates and propose models to explain the data. Experiments will be designed to test the models and to carry out experiments to support or disprove the proposed models. It is essential to advance our understanding of the structure of bacteriorhodopsin, if possible, in order to provide the best developed framework for interpretation of the NMR data.

We have made substantial progress in all of the first five steps and are hard at work on the sixth.

1. We have made excellent progress in studies of 4-C-13 aspartic acid in bacteriorhodopsin. These experiments provide evidence for four different types of sites within bR, including a protonated, an unprotonated, and two unusually H-bonded aspartic acid residues. The initial C-13 aspartic acid incorporation used the method of Eisenstein et al. (4) and led to extensive proteolytic modification of surface loops of bacteriorhodopsin (Ross, Keno, Nakanishi and Dratz, unpublished data). Therefore, we have been optimizing the in vivo biosynthetic incorporation of C-13 aspartic acid into intact bacteriorhodopsin.

   We are systematically optimizing growth conditions for producing the large amounts of 13C-asp labeled bR needed for solid state NMR studies (40-50 mg protein per experiment). Although

Halobacteria have a transport system for aspartate, addition of asp to the culture medium is not required for growth since the cells can synthesize asp. Subsequent metabolism could cause the 13C-label to be scrambled into other amino acids. Some doubt exists in the literature concerning the biosynthesis in halobacteria of the amino acids derived from aspartate, although space will not be taken to review this. Asp can serve as the immediate precursor of asparagine, lysine, methionine, and threonine. Threonine is the precursor of isoleucine via enzymes shared with the biosynthesis of valine. Our experiments with two strains of H. halobium R1 and JW-3 indicate that lysine, methionine, and isoleucine are all absolutely essential and are not produced significantly from added Asp. Threonine is neither required nor inhibitory. Glutamate and asparagine are also not required. However, eliminating both from the media results in a slight decrease in total cell production.

Aspartate also serves as a precursor for the biosynthesis of purines and pyrimidines. This can lead to two problems. Some carbon-flow will be directed to the nucleotides resulting in loss of 13C-label. More seriously, the utilization of the asp amide group in the purine bases yields fumarate as a by-product. Fumarate can enter the TCA cycle with 13C-label then appearing in other amino acids. We have found that the cell growth rate is greatly slowed when folic acid is omitted from the synthetic medium. Folic acid is required as a cofactor for dihyrdrofolate reductase, a key enzyme in both the purine and pyrimidine biosynthetic pathways. Therefore, the flow of asp into nucleotides and fumarate is suppressed by omitting folic acid. Full growth rate and levels are restored by adding nucleotides to the medium lacking folic acid. Asp can also enter the TCA cycle directly by conversion to oxaloacetate. This carbon-flow problem can be minimized by providing the cells with a high concentration of a TCA cycle intermediate such as α-ketoglutarate (2) or malate. We are now growing H. halobium with added 13C-asp and a trace amount of 14C-asp in folate free, nucleotide supplemented media with malate. Amino acid analysis of purple membrane isolated from these cultures has been used to determine the extent and selectivity of 14C-asp incorporation into bR (Figure 1).

2. We have discovered that most preparations of purple membrane are not composed of a single species of bacteriorhodopsin. In reality, they contain 4-10 different bacteriorhodopsin species (5-7). The major, well-known, mature bacteriorhodopsin protein (MW 26000 kD) can be as little as 65% of the total (5). During growth, the bacteria initially synthesize bR with an extra 13-amino acid leader sequence. In eukaryotic cells, protein leader sequences are thought to be removed by proteolytic processing in one step. However, we have found that the bacteriorhodopsin leader sequence is removed in three to four steps producing at least four to five separate bR species with different amino acid sequences. SDS polyacrylamide gel electrophoresis separates bR

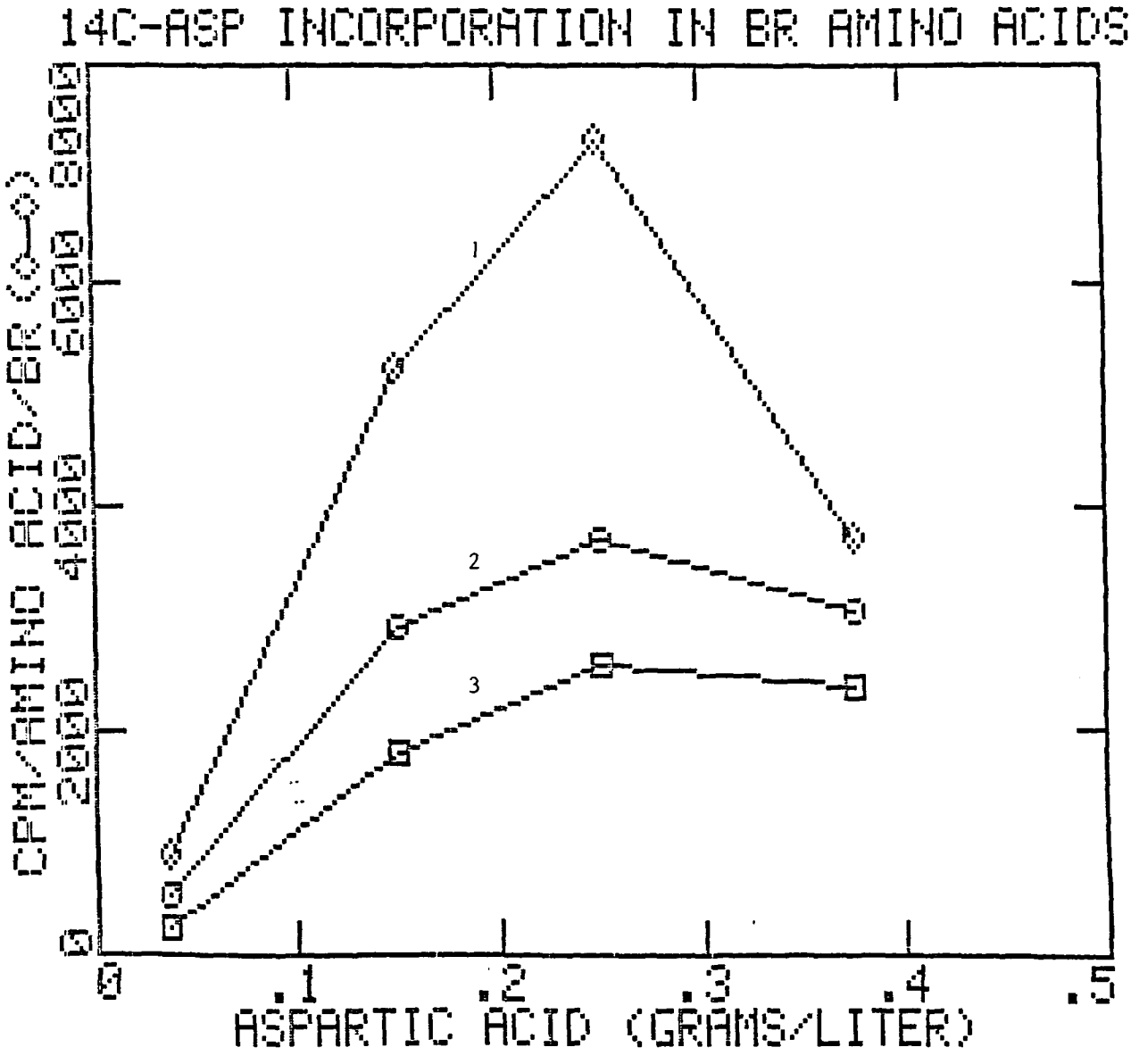

Figure 1. Incorporation of isotopic label derived from externally added 14C-aspartic acid into the amino acid residues of bacteriorhodopsin. Uniformly labeled 14C-asp was added to the synthetic growth medium of H. halobium JW-3 at the concentrations indicated. The cultures were grown for five days and the purple membrane fraction prepared by standard techniques. After delipidation of the isolated purple membrane, the bacteriorhodopsin was hydrolyzed in 6 N HCl at 110°C for 48 hr. The resulting amino acid mixture was derivatized with phenylisothiocyanate and separated using a reverse phase HPLC column. Fractions were collected and counted for radioactivity. The counts were corrected for the initial specific activity of the added aspartic acid, and normalized to the relative amount of each amino acid found both in the HPLC chromatogram and in the known amino acid content of bacteriorhodopsin. Radioactivity was found only in the fractions corresponding to aspartic acid (1), glutamic acid (2) and threonine (3).

into four different molecular weight species (5,7). The N-terminal amino sequences of these different molecules have been deduced (5). Isoelectric focussing (6,8) shows that several of these species have different surface charges. Papain proteolysis of the C-terminal amino acids of bacteriorhodopsin has been used to calibrate a 0.3 pH unit IEF shift associated with removal or addition of a single charge on the surface of the protein (6,8). IEF also shows each of the species occurs as a doublet, which we propose is due to a conformational equilibrium between different forms of bacteriorhodopsin. We have now identified growth conditions which produce a single molecular weight species as determined by SDS PAGE.

Recent reports indicate that cations are required by bR in order to maintain native function as a light-driven proton pump (9,10). There is no apparent specificity for the cation species which can reconstitute native activity. Distinct cation ion binding sites have been observed using x-ray diffraction difference density maps for lead minus calcium reconstituted membranes (11) We have recently proposed that a cation-binding site could be formed by bR residues asp-102, asp-104, gln-105, thr-107, a bound water molecule, and glu-161 or -166 (12). These residues occur in a region of striking sequence homology with the EF-hand domain of the second calcium-binding site in calmodulin.

Molecular modeling and conformational energy calculations have supported the identification of this essential cation binding site on the cytoplasmic membrane surface of bacteriorhodopsin (Helgerson, Klein, Langridge and Dratz, manuscript in preparation). During this work, we have also developed a new algorithm for detecting and identifying cation binding sites in proteins (Helgerson and Dratz, manuscript in preparation). These results should prove to be extremely useful in the ultimate interpretation of the NMR data as to the pumping mechanism of bacteriorhodopsin.

3. We have had to work out several technical details necessary to obtaining suitable solid state C-13 and F-19 spectra from these wet biological membrane systems. The first was to design and build sample holders that can spin at the high speeds needed (2-7 KHz). These sample holders do not leak the wet samples at ambient temperature and retain their balance when frozen samples are used. This has been accomplished satisfactorily, and improvements are continually being tested.

   Another major problem has been Rf heating of the wet membrane samples during the NMR pulsing sequence. Measurement of this potentially great Rf sample heating has been difficult in solid state NMR. We have developed a new method using a C-13 enriched acetate complex of the rare earth samarium. Samarium acetate is known to have a temperature dependent spectrum. We have used this technique in situ by using epoxy to seal a small amount of the samarium acetate in the bottom of a sample spinner. The wet membrane pellet sample is then loaded on top of the sealed samarium acetate. Temperature dependent shifts in the CPMAS

spectrum of samarium acetate versus the pulse sequence duty cycle are used to detect any sample heating and to measure the sample temperature during NMR experiments. Significant decreases in the duty cycle (to 1%, 30 msec/3 sec) are required to maintain <5% sample heating and appropriate temperature control.

Solid state F-19 NMR spectra measurements are under test development. I expect to report on them at the ONR Membrane Electro-Chemistry Meeting in November. F-19 is attractive because it is an extremely sensitive probe for environmental changes while not impairing function in most cases.

4. We have used the Herzfeld-Berger program (13) to deduce full chemical shift anisotropy patterns from intermediate spinning speed solid state NMR data. This program is run on our VAX Network computer and has been used successfully for obtaining the CSA values from spinning sideband intensity data in several model compounds.

5. We have refined methods for isolating enriched amounts of the key K and M photointermediates by low temperature trapping methods. We are now developing procedures to isolate the NMR spectra of the O photointermediate by synchronizing laser excitation pulses with NMR data acquisition.

6. The phase of this research where a wider range of data will be acquired is just beginning. In the process of reaching this crucial state, we have made unexpected progress in deducing the three-dimensional, atomic resolution structure of bacteriorhodopsin. If the three-dimensional atomic structure of bacteriorhodopsin can be obtained, it will greatly aid the interpretation of the NMR data in terms of detailed mechanistic information.

We had previously made excellent progress in obtaining well ordered, three-dimensional crystals of bacteriorhodopsin. However, they are too small for structure determination by x-ray diffraction (Miercke, Stroud and Dratz, unpublished data). In our attempts to obtain preparations of bacteriorhodopsin with a single, homogenous amino acid sequence, we have also found conditions for growing larger crystals. Proteolytic trimming of bacteriorhodopsin to a single molecular weight species enhances the growth of larger crystals. Rigorous purification of the detergent used for solubilization and in crystallization of bR also leads to larger crystal formation. It is anticipated that combining these two key steps will lead to the production of sufficiently large crystals and, in collaboration with Prof. Robert Stroud's group at UC San Francisco, to the determination of the three-dimensional structure of bacteriorhodopsin at atomic resolution. Methods developed here should prove useful for the crystallization of other membrane proteins and for revealing their mechanisms of action using solid state NMR spectroscopy.

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(1) L. J. W. Miercke, P. E. Ross, R. M. Stroud and E. A. Dratz, Purification of bacteriorhodopsin and characterization of mature and partially processed forms. Submitted to The Journal of Biological Chemistry.

(2) L. J. W. Miercke, R. M. Stroud and E. A. Dratz, Preparative purification of bacteriorhodopsin by high-performance size exclusion chromatography. Submitted to Analytical Biochemistry.

(3) P. E. Ross, S. L. Helgerson, L. J. W. Miercke and E. A. Dratz, Studies of multiple ioselectric forms of bacteriorhodopsin (1988) Biophys. J. 53:439a.

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